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Accordance and concordance of PCR and NASBA followed by oligochromatography for the molecular diagnosis of *Trypanosoma brucei* and *Leishmania*

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Summary

OBJECTIVE To evaluate the repeatability and reproducibility of four simplified molecular assays for the diagnosis of *Trypanosoma brucei* spp. or *Leishmania* ssp. in a multicentre ring trial with seven participating laboratories.
METHODS The tests are based on PCR or NASBA amplification of the parasites nucleic acids followed by rapid read-out by oligochromatographic dipstick (PCR-OC and NASBA-OC).
RESULTS On purified nucleic acid specimens, the repeatability and reproducibility of the tests were *Tryp*-PRC-OC, 91.7% and 95.5%; *Tryp*-NASBA-OC, 95.8% and 100%; *Leish*-PCR-OC, 95.9% and 98.1%; *Leish*-NASBA-OC, 92.3% and 98.2%. On blood specimens spiked with parasites, the repeatability and reproducibility of the tests were *Tryp*-PRC-OC, 78.4% and 86.6%; *Tryp*-NASBA-OC, 81.5% and 89.0%; *Leish*-PCR-OC, 87.1% and 91.7%; *Leish*-NASBA-OC, 74.8% and 86.2%.
CONCLUSION As repeatability and reproducibility of the tests were satisfactory, further phase II and III evaluations in clinical and population specimens from disease endemic countries are justified.

keywords leishmaniasis, trypanosomiais, diagnostics, nucleic acid sequence–based amplification, polymerase chain reaction, oligochromatography

Introduction

Polymerase chain reaction (PCR) and nucleic acid sequence–based amplification (NASBA) are established techniques for the detection of pathogen DNA or RNA, respectively, and standardized formats of PCR and NASBA have been developed for the diagnosis of human African trypanosomiasis and leishmaniasis targeting 18S ribosomal DNA or RNA (van der Meide *et al.* 2005; Deborggraeve *et al.* 2006, 2008; van der Medier *et al.* 2008; Mugasa *et al.* 2008).

To facilitate implementation of molecular diagnostics in laboratories in disease endemic countries, there is a simple dipstick format for the detection of amplification products, i.e. oligochromatography or OC (Deborggraeve *et al.* 2006; Claes *et al.* 2007; Mugasa *et al.* 2009). After nucleic acid amplification with either PCR or NASBA, the amplification products are allowed to migrate on the sensitized membrane of an oligochromatographic dipstick. The detection step can be performed in 5–10 min and no other equipment than a pipette and water bath are needed. Nucleic acid amplification control, internal control and migration control are incorporated in the assays. Combining PCR or NASBA with OC has led to the development of four different diagnostic tests for either trypanosomiasis or leishmaniasis: *Tryp*-PCR-OC (Deborggraeve *et al.* 2006); *Tryp*-NASBA-OC (Mugasa *et al.* 2009); *Leish*-PCR-OC (Deborggraeve *et al.* 2008) and

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Leish-NASBA-OC (Mugasa *et al.* 2010) and all tests have shown satisfactory analytical sensitivity and specificity.

Before these tests can be subjected to large-scale phase II and III evaluations with participating laboratories in disease endemic countries, they should demonstrate sufficient repeatability and reproducibility. Determining these parameters must be part of the development flow of any diagnostic test from proof-of-principle to demonstrating their utility in any diagnostic setting, but it is rarely reported. Repeatability measures the variability between measurements when performed on identical specimens by the same executor in the same laboratory and using the same equipment. Reproducibility measures the variability when identical specimens are analysed by different executors in different laboratories. However, repeatability and reproducibility cannot be measured for tests generating qualitative data such as OC tests described above. Therefore, Langton *et al.* (2002) introduced two new measures, as analogues for repeatability and reproducibility of tests generating qualitative data, i.e. accordance (ACC) and concordance (CON), respectively.

Our aim was to assess the ACC and CON of the PCR-OC and NASBA-OC assays on purified nucleic acid specimens as well as on human blood spiked with parasites in a multicentre ring trial with seven participating laboratories from Africa and Europe.

Materials and methods

Participating laboratories

The ring trial was performed by trained laboratory technicians in seven independent laboratories in six countries: Makerere University in Uganda, Institut National de Recherche Biomédicale in Democratic Republic (DR) of the Congo, Kenya Medical Research Institute in Kenya, University of Khartoum in Sudan, The Koninklijk Instituut voor de Tropen Biomedical Research in The Netherlands, Coris BioConcept and Institute of Tropical Medicine both in Belgium. The laboratories in Europe evaluated all four tests; the laboratories in Uganda and DRC evaluated the *Tryp*-PCR-OC and *Tryp*-NASBA-OC and the laboratories in Kenya and Sudan tested the *Leish*-PCR-OC and *Leish*-NASBA-OC. All participants were trained in test execution during a one-week workshop held at Makerere University (Kampala, Uganda).

Specimen preparation

In-vitro cultured *Trypanosoma brucei gambiense* (LiTat 1.3) and *Leishmania donovani* (MHOM/SD/68/S1) parasites were suspended in phosphate-buffered saline (PBS) at a concentration of 10^6 parasites per ml PBS. This suspension was used to prepare two different sets of specimens. The first set of specimens comprised serially diluted parasites in Tris-EDTA (TE) buffer containing 20 μg/ml calf thymus DNA to prevent degradation of the target DNA (dilution buffer) at concentrations of 100 000, 10 000, 1000, 100, 10 and 0 parasites per ml. The second set comprised human blood from a healthy volunteer spiked with 1000, 100, 10 and 0 *T. brucei gambiense* or *Leishmania* parasites per ml of blood. All blood specimens were prepared in triplicate. Four blood specimens from non-endemic healthy volunteers were further included in the study as negative controls.

Transportation and storage of specimens

All specimens were prepared by a single individual in a central laboratory. As cooled transport to several participants could not be guaranteed, the blood specimens were stabilized on silica before shipment. This was carried out by performing the first step in the nucleic acid extraction protocol (Boom *et al.* 1990). In brief, 200 μl of each specimen was mixed with 1.2 ml of L6 lysis buffer (50 mmTris–HCl, 5m GuSCN 20 mm EDTA, 0.1% Triton X100) and subsequently 40 μl of silica suspension was added and mixed for a further 5 min. The samples were centrifuged and the supernatant discarded, leaving behind a pellet of silica with bound nucleic acids. The specimen sets were shipped on dry ice to the various participating laboratories. All samples were coded and tested blindly by a trained technician in each of the seven laboratories.

Extraction of nucleic acids

The silica pellet was washed twice with 1 ml of L2 wash buffer (5 m GuSCN, 100 mm Tris–HCl [pH 6.4] supplied by the central laboratory to trial participants), twice with 1 ml of 70% ethanol, and once with 1 ml of acetone. The pellet was dried at 56 °C for 5 min after which the nucleic acids were eluted in 50 μl TE buffer during 5-min incubation at 56 °C. The eluted nucleic acids were stored at −20 °C until amplification.

Ring trial study

The *Trypanosoma* specimen set was analysed with the *Tryp*-NASBA-OC and *Tryps*-PCR-OC; while the *Leishmania* specimen set was analysed with the *Leish*-NASBA-OC and *Leish*-PCR-OC. With the specimen set and necessary test reagents, the participating laboratories also received a test report sheet, questionnaire and standard operating procedures for nucleic acid extraction as

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described by Boom *et al.* (1990), and the PCR-OC and NASBA-OC execution protocols as described by Deborggraeve *et al.* (2006) Deborggraeve *et al.* (2008) and Mugasa *et al.* (2009, 2010). The laboratories further purified the nucleic acids from the blood specimens and analysed the blood specimens once and the nucleic acid dilutions in PBS thrice. All specimens were tested blindly. Test results were reported to the central laboratory for statistical analysis.

Statistical analysis

ACC, defined as the average chance of finding the same result for two identical specimens analysed in the same laboratory, and CON, defined as the average chance of finding the same result for two identical specimens analysed in different laboratories, of *Tryp*-PCR-OC, *Tryp*-NASBA-OC, *Leish*-PCR-OC and *Leish*-NASBA-OC were estimated in a random framework by the formulae described by van der Voet and van der Raamsdon (2004): $ACC_r = (1/L)\ \Sigma_i\ (p_{0,i}^2+p_{1,i}^2)$ and $CON_r = P_0^2+P_1^2$, in which $p_{0,i}^2$ and $p_{1,i}^2$ were defined as the squared proportion of *Tryp*-PCR-OC, *Tryp*-NASBA-OC, *Leish*-PCR-OC and *Leish*-NASBA-OC negative and positive results, respectively, for each analysis i and where P_0^2 and P_1^2 were defined by the equations $P_0^2 = (1/L)\ \Sigma_{i=1}\ p_{0,i}$ and $P_1^2 = (1/L)\ \Sigma_{i=1}\ p_{1,i}$ with L the number of laboratories in the trial. Around the ACC and CON estimates, 95% confidence intervals (CI) were quantified by bootstrapping (Davison & Hinkley 1997).

Results

An overview of the test results at the different laboratories is presented in Tables 1 (*Tryp*-PCR-OC and *Leish*-PCR-OC) and 2 (*Tryp*-NASBA-OC, and *Leish*-NASBA-OC).

Table 1 Results of the *Tryp* and *Leish* PCR-OC tests on the specimen sets in the seven laboratories participating in the study

		no of positive *Tryp*-OC-PCR tests at laboratory					no of positive *Leish*-OC-PCR tests at laboratory				
Specimen	no of repetitions	1	2	3	4	5	1	2	3	6	7
Trypanosoma brucei gambiense NA in TE buffer											
100 000 parasites/ml	3	3	3	3	3	3					
10 000 parasites/ml	3	3	3	3	3	3					
1000 parasite/ml	3	3	3	3	3	3					
100 parasite/ml	3	3	3	3	1	3					
10 parasite/ml	3	2	2	0	0	0					
0 parasite/ml	3	0	0	0	0	0					
Leishmania donovani NA in TE buffer											
100 000 parasites/ml	3						3	3	3	3	Inv
10 000 parasites/ml	3						3	3	3	3	Inv
1000 parasite/ml	3						3	3	3	2	Inv
100 parasite/ml	3						2	3	3	2	Inv
10 parasite/ml	3						2	0	0	2	Inv
0 parasite/ml	3						0	0	0	0	Inv
Trypanosoma brucei gambiense parasites in blood											
1000 parasites/ml	3	3	3	3	3	3					
100 parasites/ml	3	3	3	2	2	3					
10 parasites/ml	3	3	3	0	1	2					
0 parasite/ml	3	1	0	0	1	1					
Leishmania donovani parasites in blood											
1000 parasites/ml	3						3	3	2	3	Inv
100 parasites/ml	3						3	3	3	3	Inv
10 parasites/ml	3						2	3	2	2	Inv
0 parasite/ml	3						0	0	2	0	Inv
Blood from healthy donors											
Donor 1	1	0	1	0	1	0	0	0	0	0	Inv
Donor 2	1	0	0	0	1	0	0	0	0	0	Inv
Donor 3	1	0	0	0	1	0	0	0	0	0	Inv
Donor 4	1	0	0	0	0	0	0	0	1	0	Inv

no, number; NA, nucleic acids; TE, Tris-EDTA; Inv, invalid; the DNA dilution series in TE buffer were provided as one series and tested in triplicate; the parasite dilution series in blood were provided in triplicate and tested one time.

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The *Leish*-PCR/NASBA-OC results of one laboratory were excluded from the data analysis as unexplained high numbers of invalid results were observed at this laboratory, probably because of incorrect test execution. A test result is considered invalid when both the test lines as the negative internal control line remain negative.

ACC and CON were calculated for the four tests on the nucleic acids dilution series in PBS and on the parasite dilution series in blood separately and are presented in Table 3.

Discussion

The evaluation of molecular diagnostics (and even diagnostics in general) is often neglected. It is important to estimate the repeatability (ACC) and reproducibility (CON) of a new test prior to further phase II and III evaluation studies (which should be performed in other laboratories than the laboratory where the test was developed). The present study reports on a multi-centre evaluation study of two molecular assays in seven laboratories on two continents. Our study's set-up and data analysis are of interest of many researchers working on diagnostic test development. The presented work can be an example for researchers wishing to develop new diagnostics for neglected tropical diseases, and it highlights some parameters that warrant control.

Our main aim was to validate inter (ACC) and intra (CON) laboratory performance of the *Tryp*-PCR-OC, *Tryp*-NASBA-OC, *Leish*-PCR-OC and *Leish*-NASBA-OC tests. When analysing purified nucleic acid specimens, the four tests showed an ACC and CON above 95% and 90%, respectively. The ACC and CON were below 90% when blood specimens were analysed. This is not surprising as

Table 2 Results of the *Tryp* and *Leish* NASBA-OC tests on the specimen sets in the seven laboratories participating in the study

		no of positive *Tryp*-NASBA-OC tests at laboratory					no of positive *Leish*-NASBA-OC tests at laboratory				
Specimen	no of repetitions	1	2	3	4	5	1	2	3	6	7
Trypanosoma brucei gambiense NA in TE buffer											
100 000 parasites/ml	3	3	3	3	3	3					
10 000 parasites/ml	3	3	3	3	3	3					
1000 parasite/ml	3	3	3	3	3	3					
100 parasite/ml	3	3	3	3	3	3					
10 parasite/ml	3	3	3	3	3	0					
0 parasite/ml	3	0	0	0	0	0					
Leishmania donovani NA in TE buffer											
100 000 parasites/ml	3						3	3	3	2	Inv
10 000 parasites/ml	3						3	3	3	2	Inv
1000 parasite/ml	3						3	3	3	2	Inv
100 parasite/ml	3						3	0	0	2	Inv
10 parasite/ml	3						3	0	0	0	Inv
0 parasite/ml	3						0	0	0	0	Inv
Trypanosoma brucei gambiense parasites in blood											
1000 parasites/ml	3	3	3	3	3	3					
100 parasites/ml	3	3	3	3	3	3					
10 parasites/ml	3	1	0	0	2	2					
0 parasite/ml	3	0	0	0	2	0					
Leishmania donovani parasites in blood											
1000 parasites/ml	3						3	3	3	3	Inv
100 parasites/ml	3						3	1	2	3	Inv
10 parasites/ml	3						3	0	0	1	Inv
0 parasite/ml	3						0	0	0	0	Inv
Blood from healthy donors											
Donor 1	1	0	0	0	0	0	0	0	0	0	Inv
Donor 2	1	0	0	0	1	0	0	0	0	0	Inv
Donor 3	1	0	0	0	0	0	0	0	0	0	Inv
Donor 4	1	0	0	0	0	0	0	0	0	0	Inv

no, number; NA, nucleic acids; TE, Tris-EDTA; Inv, invalid; the DNA dilution series in TE buffer were provided as one series and tested in triplicate; the parasite dilution series in blood were provided in triplicate and tested one time.

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Table 3 Overview of the accordance and concordance of the *Trypanozoon* and *Leishmania* OligoC-TesT on DNA and blood specimens analysed during the ring trial

Test	Accordance % (95% CI)	Concordance % (95% CI)
Dilution series of DNA		
Tryps-PCR-OC test	95.5 (92.6–98.5)	91.7 (87.0–97.9)
Leish-PCR-OC test	98.1 (94.4–100)	95.9 (91.7–100)
Dilution series of RNA		
Tryps-NASBA-OC test	100	95.8 (92.0–100)
Leish-NASBA-OC test	98.2 (94.4–100)	92.3 (87.0–100)
Dilution series of parasites in blood		
Tryps-PCR-OC test	86.6 (75.6–97.8)	78.4 (68.5–91.1)
Leish-PCR-OC test	91.7 (88.9%–97.2)	87.1% (80.9–96.2)
Tryps-NASBA-OC test	89.0 (82.2–95.6)	81.5 (72.4–90.7)
Leish-NASBA-OC test	86.2 (77.8–94.4)	74.8 (67.0–89.2)

Accordance, intralaboratory repeatability; Concordance, interlaboratory reproducibility; 95% CI, 95% confidence interval by bootstrapping.

the nucleic acids had to be extracted from the blood specimens by the participating laboratories, leading to higher chances of intra and interlaboratory inconsistencies. In addition, the variation in test results was predominantly observed in the low parasite range of 1–0 parasites/ml blood. As this parasite concentration is close to the analytical sensitivity of the tests (Deborggraeve *et al.* 2006, 2008; Mugasa *et al.* 2009, 2010), slight variations in equipment and operating procedures may have a major influence on the results obtained with these low parasite concentrations.

Occasional false positive results were observed in the blood specimens from healthy European controls. Although cross-reaction of the tests with human DNA or RNA cannot be excluded, carry-over contamination during DNA extraction from the blood specimens is more likely for two reasons. First, no false positive results were observed in the specimen sets containing DNA. Secondly, there is the observation that three of the five false positive results for DNA were obtained in the same laboratory. The results of one laboratory analysing the *Leish*-PCR/NASBA-OC tests had to be excluded because very high numbers of invalid results were generated, most probably because of faulty test execution. Similar problems were not encountered at the other laboratories.

To our knowledge, this is the first study evaluating the ACC and CON of molecular diagnostic tests for human African trypanosomiasis and leishmaniasis on blood samples. The ACC and CON of the Tryp-PCR-OC have already been estimated in a multicentre ring trial with six participating laboratories on purified DNA specimens, reported by Claes *et al.* (2007). In that study, the test showed an ACC of 88.7% (95%CI: 84.4%-92.5%) and a CON of 88.1% (95%CI: 84.3%-92.3%), slightly lower than the results obtained in this study.

In conclusion, the ACC (repeatability) and CON (reproducibility) of the *Tryp*-PCR-OC, *Leish*-PCR-OC, *Tryp*-NASBA-OC and *Leish*-NASBA-OC tests were successfully estimated by (i) a multicentre ring trial with several European and African participants and (ii) data analysis of the qualitative test results in a random framework as described by van der Voet and van der Raamsdon (2004). All four tests demonstrated to be repeatable and reproducible and can undergo further phase II and III evaluations in variable laboratory settings in disease endemic countries.

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